Volume 203, number 2 FEBS 3875 July 1986

Cell alkalinization is not necessary and increased sodium influx is not sufficient for stimulated superoxide production

T.F.P. Molski, C. Ford*, S.J. Weisman* and R.I. Sha'afi

Departments of Physiology and **Pediatrics, University of Connecticut Health Center, Farmington, CT 06032, USA*

Received 26 April 1986

Preincubation of rabbit neutrophils for 5 min with the protein kinase C inhibitor H7 causes inhibition of the rise in intracellular pH but not the increase in Na^+ influx or stimulated oxidative burst produced by the chemotactic factor formyl-methionyl-leucyl-phenylalanine. On the other hand, the stimulated superoxide production, but not the increase in Na^+ influx produced by phorbol 12-myristate 13-acetate, is inhibited by H7. The effect is more pronounced on the rate than the extent of the stimulated superoxide release. Furthermore, cell acidification produced by the phorbol ester but not by the chemotactic factor is decreased in the presence of H7. These results suggest that (i) most of the stimulated Na^+ influx is not coupled to H^+ efflux, (ii) in the case of the chemoattractant, the rise in intracellular pH is not necessary for stimulated superoxide production, (iii) the increase in Na^+ influx, in the case of the phorbol ester, is not sufficient for the stimulation of the oxidative burst, and (iv) the sources of the H^+ responsible for the stimulated pH drop are the various metabolic activities of the cell, including NADPH oxidation and activation of the hexose monophosphate shunt.

H7 *Ion flux* *Protein kinase C* *Superoxide*

1. INTRODUCTION

The addition of the chemotactic factor fMet-Leu-Phe to neutrophils activates several cell functions and profoundly modifies the permeability characteristics of the plasma membranes of these cells [1–17]. Among the most dramatic of the permeability changes initiated by fMet-Leu-Phe are rapid increases in Na^+ and H^+ fluxes.

It has been suggested, in analogues to other cell types, that the stimulus-induced increases in Na^+ influx and H^+ efflux are mediated by the Na^+/H^+ antiport system which is activated, in part but not solely, by protein kinase C. It has also been hypothesized that the rise in intracellular pH and increase in Na^+ influx produced by chemotactic stimuli are important for stimulated superoxide production [14–17]. The evidence linking the Na^+/H^+ antiport to the increase in superoxide generation is based on the demonstration that amiloride, a known inhibitor of this system, inhibits both stimulated Na^+ influx, H^+ efflux and superoxide production produced by fMet-Leu-Phe [11,12,14–17]. The role of protein kinase C in the regulation of the Na^+/H^+ antiport and superoxide production is based on the importance of protein kinase C in cell activation [18] and on the finding that the addition of phorbol 12-myristate 13-acetate (PMA) to neutrophils increases Na^+ influx, H^+ efflux and superoxide production [9,11,12,17,19].

The present studies were undertaken to investigate the relationship among stimulated Na^+ influx, H^+ efflux and superoxide production. We have found, using the protein kinase C inhibitor, H7 [20,21], which inhibits both the agonist and antagonist properties of PMA [22,23], that (i) most of the increase in Na^+ influx produced by fMet-Leu-Phe can be dissociated from the corresponding increase in H^+ efflux, (ii) the rise in intracellular pH but not the corresponding increase in superoxide production produced by fMet-Leu-Phe is inhibited by H7, and (iii) the PMA-induced

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00145793/86/$3.50

changes in superoxide production and cell acidification but not the stimulated Na^+ influx are inhibited by H7.

2. MATERIALS AND METHODS

Rabbit neutrophils (4–16 h exudate) were collected, washed and resuspended in Hanks' balanced salt solution buffered with 10 mM Hepes as described [19]. The buffered solution contained 1 mg/ml albumin and no added magnesium. The cells were suspended at 10^7 cells/ml. When H7 was used, the cells were incubated with H7 for 5 min before stimulation.

Changes in the intracellular pH of the neutrophils were measured by following the distribution of the weak acid 5,5′-dimethyloxazolidine-2,4-dione (DMO) as in [9]. The cells (10^7 cells/ml) were incubated with [^{14}C]DMO for 1 min, then the chemoattractant was added and the distribution of the label was followed with time. We have also used the pH-sensitive fluorescent dye 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). In these experiments the extracellular medium pH was 7.0.

Na^+ influx was measured using the rapid sampling silicone oil technique detailed in [13]. Radiolabelled sodium and the desired stimuli were added simultaneously and the amount of radioactivity associated with the cells was measured with time.

Superoxide production was determined using the method of Cohen and Chovaniec [24]. The continuous measurement of O_2^--dependent cytochrome *c* reduction was performed at 37°C in a Lambda 3B double-beam spectrophotometer equipped with a 3600 Data Station (Perkin-Elmer, Norwalk, CT). Briefly, isolated PMNs were suspended at 5×10^6/ml in Krebs-Ringers-phosphate with glucose (pH 7.3) plus 119 μM cytochrome *c*. The reference cuvette contained 0.02 mg/ml of superoxide dismutase. The various stimuli were introduced simultaneously to both the reference and experimental cuvettes and the reduction of cytochrome *c* was followed spectrophotometrically at 550 nm. The release of O_2^- was determined as the change in absorbance from baseline after 10 min. This was converted to nmol reduced cytochrome *c* by use of the molar extinction coefficient 21.1×10^3 $M^{-1} \cdot cm^{-1}$.

Table 1

Effect of the addition of H7 on fMet-Leu-Phe-induced changes in intracellular pH

Experimental conditions	Change in intracellular pH (mean ± SE)[a]	
	$\Delta pH_{i,1}$	$\Delta pH_{i,7}$
Control	0.00 ± 0.01	−0.01 ± 0.01
+ fMet-Leu-Phe (10^{-9} M)	−0.06 ± 0.02	0.08 ± 0.02
H7 (25 μM)	−0.02 ± 0.01	0.01 ± 0.005
H7 + fMet-Leu-Phe (10^{-9} M)	−0.07 ± 0.02	0.02 ± 0.015
+ fMet-Leu-Phe (10^{-8} M)	−0.09 ± 0.025	0.10 ± 0.025
H7 + fMet-Leu-Phe (10^{-8} M)	−0.11 ± 0.03	0.03 ± 0.015

[a] $\Delta pH_{i,1} = pH_{i,1} - pH_{i,0}$; $\Delta pH_{i,7} = pH_{i,7} - pH_{i,0}$ where $pH_{i,0}$, $pH_{i,1}$ and $pH_{i,7}$ are the values of the intracellular pH at 0 time, 1 min and 7 min, respectively. The cells were preincubated with H7 for 5 min before [^{14}C]DMO was added. Each value represents the mean ± SE of at least three separate experiments. Each experiment was carried out in duplicate

fMet-Leu-Phe, Hepes, cytochrome *c* and superoxide dismutase were purchased from Sigma (St. Louis, MO), PMA was obtained from CMC, Cancer Chemicals (Brewster, NY), and 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H7) was purchased from Seikagaku America (St. Petersburg, FL). The ^{14}C-labelled DMO and ^{22}Na were purchased from New England Nuclear (Boston, MA).

3. RESULTS AND DISCUSSION

The effect of pretreatment of rabbit neutrophils with the protein kinase C inhibitor H7 on the changes in intracellular pH produced by the chemotactic factor fMet-Leu-Phe was examined (table 1). The results summarized in this table clearly show that the chemotactic factor fMet-Leu-Phe causes a rapid decrease (acidification) followed by an increase (alkalinization) in the intracellular pH. Similar results were obtained using the pH-sensitive fluorescent dye BCECF. The initial acidification (most likely due to metabolic events of the cells) is not affected by incubating the cells for 5 min with 25 μM H7. On the other hand, the

alkalinization (most likely due to Na^+/H^+ antiport) is inhibited by H7. Note that H7 by itself has no significant effect. The results with BCECF were not reliable.

The addition of fMet-Leu-Phe to neutrophils causes an increase in Na^+ influx in addition to the rise in intracellular pH. It is commonly hypothesized, as in other cell types, that stimulated Na^+ influx is coupled to H^+ efflux through the Na^+/H^+ antiport. In order to test this hypothesis, we have investigated the effect of H7 on the increase in Na^+ influx produced by fMet-Leu-Phe. The results summarized in fig.1 clearly show that incubation of the rabbit neutrophils for 5 min with 25 μM H7, a concentration which reduces the stimulated rise in intracellular pH, does not affect the increase in Na^+ influx produced by fMet-Leu-Phe. This clear dissociation of the two stimulated fluxes (Na^+ influx and H^+ efflux) strongly suggests that most of the increase in Na^+ influx produced by fMet-Leu-Phe occurs through a pathway which is separate from the Na^+/H^+ antiport.

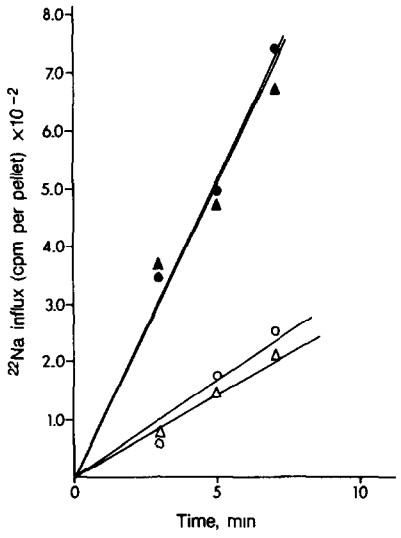

Fig.1. Effect of H7 on the time course of ^{22}Na influx in control and fMet-Leu-Phe-stimulated rabbit neutrophils. (△) Control cells, (▲) control cells stimulated with fMet-Leu-Phe (10^{-9} M), (○) H7-treated (25 μM for 5 min) cells, (●) H7-treated cells stimulated with fMet-Leu-Phe. Results are taken from a single experiment that is representative of at least three separate experiments. Each was carried out in duplicate. The pellet represents 5×10^6 cells.

Table 2

Effect of H7 on superoxide production produced by fMet-Leu-Phe and PMA

Experimental condition	Superoxide production (nmol/5 × 10^6 cells per 10 min)	
	Control	+ H7 (25 μM)[a]
+ fMet-Leu-Phe (10^{-9} M)	5.5 ± 0.5	8.0 ± 0.5
+ fMet-Leu-Phe (10^{-8} M)	22.5 ± 1.0	28.4 ± 1.0
+ PMA (100 ng/ml)	54.1 ± 2.0	24.0 ± 1.5

[a] The cells were incubated with H7 for 5 min before the stimulus was added. The reaction was carried out for 10 min after the addition of the stimulus. Each value represents the mean ± SE of at least two separate experiments. H7 by itself has small (< 10% stimulation) effect

To examine the relationship between the rise in intracellular pH and the increase in superoxide production, we have investigated the effect of pretreatment of the cells with H7 on production of superoxide produced by fMet-Leu-Phe and PMA (table 2). The data in this table clearly show that, unlike the rise in intracellular pH produced by fMet-Leu-Phe, the addition of H7 has no inhibitory effect on superoxide production produced by the same stimulus. In fact, a small potentiation by H7 of the oxidative burst response to the agonist can be observed. This indicates that the two responses (rise in pH and O_2^- production) initiated by the chemoattractant can be clearly dissociated. On the other hand, the addition of 25 μM H7 for 5 min inhibits, by more than 50%, superoxide production produced by PMA. It must be clearly pointed out that the inhibition is more pronounced on the rate and not the extent of superoxide production (i.e. H7 slows the reaction).

Next we investigated the effect of H7 on the PMA-induced increase in Na^+ influx. The results summarized in fig.2 show that pretreatment of the cells with 25 μM H7 for 5 min, conditions which significantly reduced superoxide release, have no effect on stimulated Na^+ influx produced by PMA.

It has been suggested that the stimulus-induced drop in intracellular pH is due to NADPH oxida-

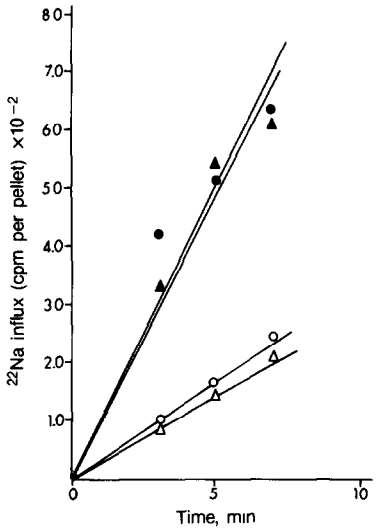

Fig.2. Effect of H7 on the time course of ^{22}Na influx in control and PMA-stimulated cells. (△) Control cells, (▲) control cells stimulated with PMA (100 ng/ml), (○) H7-treated (25 μM for 5 min) cells, (●) H7-treated cells stimulated with PMA. Results are taken from a single experiment that is representative of at least three separate experiments. Each was carried out in duplicate. The pellet represents 5 × 10^6 cells.

tion and/or the associated increase in the hexose monophosphate shunt activity [25]. To examine this point, we tested the effect of the protein kinase C inhibitor H7 on the decrease in pH_i following stimulation by PMA and fMet-Leu-Phe. The results summarized in table 3 clearly show that both O_2^- production and the acidification produced by PMA but not by fMet-Leu-Phe are reduced in the presence of H7.

The results presented above make several separate, though interrelated, points.

(1) The rise in intracellular pH produced by fMet-Leu-Phe is significantly diminished by the protein kinase C inhibitor H7. This suggests that the protein kinase C system, through the phosphorylation of one or more proteins, is involved in cell alkalinization, possibly through the regulation of the Na^+/H^+ antiport.

(2) H7, at a concentration which reduces the rise in intracellular pH, has little effect on the increase in Na^+ fluxes produced by fMet-Leu-Phe. This clear dissociation of the two fluxes strongly suggests that most of the stimulated Na^+ influx occurs through a pathway separate from the Na^+/H^+ antiport.

(3) H7, at a concentration which reduces the rise in intracellular pH, has no inhibitory effect on the stimulated superoxide production by fMet-Leu-Phe. In fact, a small but significant increase is commonly observed. Gerard et al. [26] have very recently demonstrated that the stimulation by PMA of the oxidative metabolism of human neutrophils, but not by fMet-Leu-Phe, could be inhibited by an

Table 3

Effect of H7 on the drop in intracellular pH and superoxide production produced by fMet-Leu-Phe and PMA

Conditions	Cell acidification $\Delta pH_{i,2}^a$		Superoxide production[b]	
	Control	+ H7	Control	+ H7
No addition	−0.01 ± 0.01	−0.02 ± 0.01	0.0	0.2 ± 0.1
+ fMet-Leu-Phe (10^{-9} M)	−0.17 ± 0.03	−0.20 ± 0.04	5.5 ± 0.5	8.0 ± 0.5
+ PMA (100 ng/ml)	−0.13 ± 0.03	−0.08 ± 0.03	54.1 ± 2.0	24.0 ± 1.5

[a] $\Delta pH_{i,2} = pH_{i,2} - pH_{i,0}$ where $pH_{i,0}$ and $pH_{i,2}$ are the values of intracellular pH at 0 and 2 min respectively. The changes are expressed as pH units. Each value represents the mean ± SE of at least two separate experiments. Each experiment was carried out in duplicate. The cells were incubated with H7 (25 μM) for 5 min and amiloride (1 mM) for 2 min before they were stimulated. Amiloride was used to enhance acidification

[b] The values of superoxide production are expressed as nmol/5 × 10^6 cells per 10 min. The cells were incubated with H7 (25 μM) for 5 min

analogue of H7, compound C-1. This clear dissociation, in the case of fMet-Leu-Phe, between the rise in intracellular pH and oxidative burst, strongly suggests that the former response is not necessary for the increase in the oxidative metabolism produced by this chemoattractant.

(4) H7, at a concentration which inhibits the increase by PMA in the oxidative burst by more than 50%, does not inhibit the stimulated Na^+ influx produced by the same stimulus. In the case of PMA, this clear dissociation between the two responses strongly suggests that the stimulated Na^+ influx alone is not sufficient to trigger superoxide production.

(5) The most likely sources of the H^+ responsible for the stimulated pH drop are the various metabolic activities of the cell and the biochemical changes that ensue following stimulation. These include NADPH oxidation, activation of the hexose monophosphate shunt, lipid remodeling, increased ATPase activities and others. This conclusion is based on several experimental findings. (i) The decrease in intracellular pH is associated with a net H^+ equivalent efflux [25]. (ii) Neutrophils obtained from patients with chronic granulomatous disease (CGD), unlike cells from normal donors, do not display acidification when stimulated with PMA in Na^+-free or amiloride-containing media [25]. (iii) PMA-induced acidification is absent in neutrophils obtained from normal donors when NADPH oxidation and/or the hexose monophosphate shunt pathways are blocked by using alkylating reagents or deoxyglucose [25]. (iv) Superoxide production and acidification produced by PMA, but not by fMet-Leu-Phe, are inhibited by the protein kinase C inhibitor H7.

(6) The finding that H7 does not inhibit the increase in Na^+ influx produced by PMA represents the only case where a protein kinase C inhibitor does not affect PMA action. All of the reported agonist and antagonist properties of PMA have been shown to be inhibited by protein kinase C inhibitors [23,26,27]. There are two possible explanations for this result. First, the PMA action on Na^+ influx is not mediated by protein kinase C. The observation that the stimulated Na^+ influx produced by the chemoattractant fMet-Leu-Phe is not affected by H7 is consistent with this view. However, this is unlikely since the inactive analogue 4α-phorbol 12,13-didecanoate does not increase Na^+ influx in those cells [19]. Second, there is a family of protein kinase C, some of which are not inhibited by H7.

ACKNOWLEDGEMENTS

Supported in part by NIH grants AI-13734-08 and GM-37694-09 and the Charles H. Hood Foundation (S.J.W.).

REFERENCES

[1] Sha'afi, R.I. and Naccache, P.H. (1981) in: Advances in Inflammation Research (Weissmann, G. ed.) vol.2, pp.115–148, Raven, New York.
[2] Weissmann, G., Smolen, J.E. and Korchak, H.M. (1980) N. Engl. J. Med. 303, 27–34.
[3] Wilkinson, P.C. (1982) Chemotaxis and Inflammation, 2nd edn, pp.81–104, Churchill Livingstone, Edinburgh.
[4] Romeo, D. (1982) Trends Biochem. Sci. 7, 408–411.
[5] Grzeskowiak, M., Della Bianca, V., Cassatella, M.A. and Rossi, F. (1986) Biochem. Biophys. Res. Commun. 135, 785–794.
[6] Hallett, M.B. and Campbell, A.K. (1982) Nature 295, 155–158.
[7] Andrews, P.C. and Babior, B.M. (1983) Blood 61, 333–340.
[8] Simchowitz, L., Spilberg, I. and DeWeer, P. (1982) J. Gen. Physiol. 79, 453–479.
[9] Sha'afi, R.I., Naccache, P.H., Molski, T.F.P. and Volpi, M. (1982) Intracellular pH: Its Measurements, Regulation and Utilization in Cellular Function (Nuccitelli, R. and Deamer, D.W. eds) pp.513–525, Liss, New York.
[10] Snyderman, R. and Pike, M.C. (1985) in: Contemporary Topics in Immunology (Snyderman, R. ed.) vol.14, pp.1–28, Plenum, New York.
[11] Grinstein, S. and Furuya, W. (1986) Am. J. Physiol. 250, C283–C291.
[12] Simchowitz, L. and Roos, A. (1985) J. Gen. Physiol. 85, 443–470.

[13] Naccache, P.H., Showell, H.J., Becker, E.L. and Sha'afi, R.I. (1977) J. Cell Biol. 73, 428–444.
[14] Simchowitz, L. (1985) J. Clin. Invest. 76, 1079–1089.
[15] Simchowitz, L. and Spilberg, I. (1979) J. Immunol. 123, 2428–2435.
[16] Weissmann, G., Korchak, H.M., Perez, H.D., Smolen, J.E., Goldstein, I.M. and Hoffstein, S.T. (1979) Adv. Inflamm. Res. 1, 95–112.
[17] Wright, J., Schwartz, J.H., Olson, R., Kosowsky, J.M. and Tauber, A.I. (1986) J. Clin. Invest. 77, 782–788.
[18] Nishizuka, Y. (1984) Nature 308, 693–698.
[19] Volpi, M., Naccache, P.H., Molski, T.F.P., Shefcyk, J., Huang, C.-K., Marsh, M.L., Munoz, J., Becker, E.L. and Sha'afi, R.I. (1985) Proc. Natl. Acad. Sci. USA 82, 2708–2712.
[20] Kawamoto, S. and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 125, 258–264.
[21] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[22] Tohmatsu, T., Hattori, H., Nagao, S., Ohki, K. and Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. 134, 868–875.
[23] Sha'afi, R.I., Molski, T.F.P., Huang, C.-K. and Naccache, P.H. (1986) submitted.
[24] Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1081–1087.
[25] Grinstein, S., Furuya, W. and Biggar, W.D. (1986) J. Biol. Chem. 261, 512–514.
[26] Gerard, C., Mcphail, L., Marfat, A., Stimler-Gerard, N., Bass, D.A. and McCall, C.E. (1986) J. Clin. Invest. 77, 61–65.
[27] Vara, F. and Rozengurt, E. (1985) Biochem. Biophys. Res. Commun. 130, 646–653.